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04-13-01

Exhibit E

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Dated 8 December 2000

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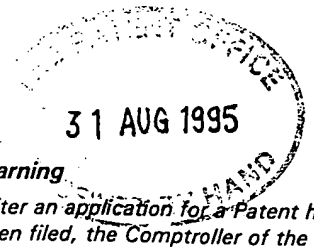


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The Patent Office

# Request for grant of a Patent

## Form 1/77

**Patents Act 1977**

## 1 Title of invention

1 Please give the title of the invention

BIOLOGICAL MANIPULATION

## 2 Applicant's details

☐ **First or only applicant**

2a If you are applying as a corporate body please give:

Corporate name
ROSLIN INSTITUTE (EDINBURGH)

Country UK
*(and State of incorporation, if appropriate)*

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases,** please give the following details:

Address
ROSLIN
MIDLOTHIAN

UK postcode EH25 9PS
*(if applicable)*

Country UK

ADP number
*(if known)* 06835912001

*2d, 2e and 2f:*
*If there are further applicants please provide details on a separate sheet of paper.*

❑ **Second applicant (if any)**

2d If you are applying as a corporate body please give:

Corporate name

Country
*(and State of incorporation, if appropriate)*

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Forenames

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**3**
*An address for service in the United Kingdom must be supplied*

*Please mark correct box*

## 3 Address for service details

3a Have you appointed an agent to deal with your application?

Yes [X] No [ ] ➡ *go to 3b*

⬇ ***Please give details below***

Agent's name
KILBURN & STRODE
Agent's address
30 JOHN STREET
LONDON

Postcode WC1N 2DD
Agent's ADP number 125001 ✓

*3b:*
*If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.*

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

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*(if known)*

Daytime telephone number *(if available)*

## 4 Reference number

4 Agent's or applicant's reference number *(if applicable)* AGS/P18826GB

## 5 Claiming an earlier application date

5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

*Please mark correct box*

Yes ☐ No ☒ ➡ *go to 6*

⬇ *please give details below*

❑ number of earlier application or patent number

❑ filing date

*(day month year)*

❑ and the Section of the Patents Act 1977 under which you are claiming:

*Please mark correct box*

15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

## 6 Declaration of priority

**6**

*If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number.*

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6 If you are declaring priority from previous application(s), please give:

| Country of filing | Priority application number *(if known)* | Filing date *(day,month,year)* |
|---|---|---|
| | | |

**7**

*The answer must be 'No' if:*
- *any applicant is not an inventor*
- *there is an inventor who is not an applicant, or*
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## 7 Inventorship

7 Are you (the applicant or applicants) the sole inventor or the joint inventors?

*Please mark the correct box*

Yes [ ] No [X] ➡

***A statement of Inventorship on Patents Form 7/77 willneed to be filed (see Rule 15).***

**8**

*Please supply duplicates of claim(s), abstract, description and drawing(s).*

## 8 Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application.

| | | | |
|---|---|---|---|
| Continuation sheets for this Patents Form 1/77 | | | |
| Claim(s) | 2 | Description | 28 |
| Abstract | 1 | Drawing(s) | 1 |
| ANNEX | 21 | | |

8b Which of the following documents also accompanies the application?

*Please mark correct box(es)*

| | |
|---|---|
| Priority documents *(please state how many)* | |
| Translation(s) of Priority documents *(please state how many)* | |
| Patents Form 7/77 - Statement of Inventorship and Right to Grant *(please state how many)* | |
| Patents Form 9/77 - Preliminary Examination/Search | |
| Patents Form 10/77 - Request for Substantive Examination | |

**9**

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## 9 Request

I/We request the grant of a patent on the basis of this application.

***Please sign here*** ➡

Signed Kilburn & Strode Date 31/08/1995

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# BIOLOGICAL MANIPULATION

This invention relates to the generation of animals including but not being limited to genetically selected and/or modified animals, and to cells useful in their generation.

The reconstruction of mammalian embryos by the transfer of a donor nucleus to an enucleated oocyte or one cell zygote allows the production of genetically identical individuals. This has clear advantages for both research (i.e. as biological controls) and also in commercial applications (i.e. multiplication of genetically valuable livestock, uniformity of meat products, animal management).

Embryo reconstruction by nuclear transfer was first proposed (Spemann, *Embryonic Development and Induction* 210-211 Hofner Publishing Co., New York (1938)) in order to answer the question of nuclear equivalence or 'do nuclei change during development?'. By transferring nuclei from increasingly advanced embryonic stages these experiments were designed to determine at which point nuclei became restricted in their developmental potential. Due to technical limitations and the unfortunate death of Spemann these studies were not completed until 1952, when it was demonstrated in the frog that certain nuclei could direct development to a sexually mature adult (Briggs and King, *Proc. Natl. Acad. Sci. USA* 38 455-461 (1952)). Their findings led to the current concept that equivalent totipotent nuclei from a single individual could, when transferred to an enucleated egg, give rise to "genetically identical" individuals. In the true sense of the meaning these

individuals would not be clones as unknown cytoplasmic contributions in each may vary and also the absence of any chromosomal rearrangements would have to be demonstrated.

Since the demonstration of embryo cloning in amphibians, similar techniques have been applied to mammalian species. These techniques fall into two categories: 1) transfer of a donor nucleus to a matured metaphase II oocyte which has had its chromosomal DNA removed and 2) transfer of a donor nucleus to a fertilised one cell zygote which has had both pronuclei removed. In ungulates the former procedure has become the method of choice as no development has been reported using the latter other than when pronuclei are exchanged.

Transfer of the donor nucleus into the oocyte cytoplasm is generally achieved by inducing cell fusion. In ungulates fusion is induced by application of a DC electrical pulse across the contact/fusion plane of the couplet. The same pulse which induces cell fusion also activates the recipient oocyte. Following embryo reconstruction further development is dependent on a large number of factors including the ability of the nucleus to direct development i.e. totipotency, developmental competence of the recipient cytoplast (i.e. oocyte maturation), oocyte activation, embryo culture (reviewed Campbell and Wilmut in *Vth World Congress on Genetics as Applied to Livestock* **20** 180-187 (1994)).

In addition to the above we have shown that maintenance of correct ploidy during the first cell cycle of the reconstructed embryo is of major importance (Campbell *et al.*, *Biol. Reprod.* **49** 933-942 (1993); Campbell *et al.*,

*Biol. Reprod.* 50 1385-1393 (1994)). During a single cell cycle all genomic DNA must be replicated once and only once prior to mitosis. If any of the DNA either fails to replicate or is replicated more than once then the ploidy of that nucleus at the time of mitosis will be incorrect. The mechanisms by which replication is restricted to a single round during each cell cycle are unclear, however, several lines of evidence have implicated that maintenance of an intact nuclear membrane is crucial to this control. The morphological events which occur in the donor nucleus after transfer into an enucleated metaphase II oocyte have been studied in a number of species including mouse (Czolowiska *et al.*, *J. Cell Sci.* 69 19-34 (1984)), rabbit (Collas and Robl, *Biol. Reprod.* 45 455-465 (1991)), pig (Prather *et al.*, *J. Exp. Zool.* 225 355-358 (1990)), cow (Kanka *et al.*, *Mol. Reprod. Dev.* 29 110-116 (1991)). Immediately upon fusion the donor nuclear envelope breaks down (NEBD), and the chromosomes prematurely condense (PCC). These effects are catalysed by a cytoplasmic activity termed maturation/mitosis/meiosis promoting factor (MPF). This activity is found in all mitotic and meiotic cells reaching a maximal activity at metaphase. Matured mammalian oocytes are arrested at metaphase of the 2nd meiotic division (metaphase II) and have high MPF activity. Upon fertilisation or activation MPF activity declines, the second meiotic division is completed and the second polar body extruded, the chromatin then decondenses and pronuclear formation occurs. In nuclear transfer embryos reconstructed when MPF levels are high NEBD and PCC occur; these events are followed, when MPF activity declines, by chromatin decondensation and nuclear reformation and subsequent DNA replication. In reconstructed embryos correct ploidy can be maintained in

one of two ways; firstly by transferring nuclei at a defined cell cycle stage, e.g. diploid nuclei of cells in $G_1$, into metaphase II oocytes; or secondly by activating the recipient oocyte and transferring the donor nucleus after the disappearance of MPF activity. In sheep this latter approach has yielded an increase in the frequency of development to the blastocyst stage from 21% to 55% of reconstructed embryos when using blastomeres from 16 cell embryos as nuclear donors (Campbell *et al.*, *Biol. Reprod.* **50** 1385-1393 (1994)).

These improvements in the frequency of development of reconstructed embryos have as yet not addressed the question of nuclear reprogramming. During development certain genes become "imprinted" i.e. are altered such that they are no longer transcribed. Studies on imprinting have shown that this "imprinting" is removed during germ cell formation (i.e. reprogramming). One possibility is that this reprogramming is affected by exposure of the chromatin to cytoplasmic factors which are present in cells undergoing meiosis. This raises the question of how we may mimic this situation during the reconstruction of embryos by nuclear transfer in order to reprogram the developmental clock of the donor nucleus.

It has now been found that nuclear transfer into an oocyte arrested in metaphase II can give rise to a viable embryo if normal ploidy (i.e. diploidy) is maintained and if the embryo is not activated at the time of nuclear transfer. The delay in activation allows the nucleus to remain exposed to the recipient cytoplasm.

According to a first aspect of the present invention there is provided a method of reconstituting an animal

embryo, the method comprising transferring a diploid nucleus into an oocyte which is arrested in the metaphase of the second meiotic division without concomitantly activating the oocyte, keeping the nucleus exposed to the cytoplasm of the recipient for a period of time sufficient for the reconstituted embryo to become capable of giving rise to a live birth and subsequently activating the reconstituted embryo while maintaining correct ploidy. At this stage, the reconstituted embryo is a single cell.

In principle, the invention is applicable to all animals, including birds such as domestic fowl, amphibian species and fish species. In practice, however, it will be to (non-human) mammals, particularly placental mammals, that the greatest commercially useful applicability is presently envisaged. It is with ungulates, particularly economically important ungulates such as cattle, sheep, goats, water buffalo, camels and pigs that the invention is likely to be most useful, both as a means for cloning animals and as a means for generating transgenic animals. It should also be noted that the invention is also likely to be applicable to other economically important animal species such as, for example, horses, llamas or rodents, e.g. rats or mice, or rabbits.

The invention is equally applicable in the production of transgenic, as well as non-transgenic animals. Transgenic animals may be produced from genetically altered donor cells. The overall procedure has a number of advantages over conventional procedures for the production of transgenic (i.e. genetically modified) animals which may be summarised as follows:

(1) fewer recipients will be required;

(2) multiple syngeneic founders may be generated using clonal donor cells;

(3) subtle genetic alteration by gene targeting is permitted;

(4) all animals produced from embryos prepared by the invention should transmit the relevant genetic modification through the germ line as each animal is derived from a single nucleus; in contrast, production of transgenic animals by pronuclear injection or chimerism after inclusion of modified stem cell populations by blastocyst injection produces a proportion of mosaic animals in which all cells do not contain the modification and may not transmit the modification through the germ line; and

(5) cells can be selected for the site of genetic modification (e.g. integration) prior to the generation of the whole animal.

It should be noted that the term "transgenic", in relation to animals, should not be taken to be limited to referring to animals containing in their germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted, duplicated, activated or modified is a transgenic animal for the purposes of this invention as much as an animal to whose germ line an exogenous DNA sequence has been added.

In embodiments of the invention in which the animal is transgenic, the donor nucleus is genetically modified. The donor nucleus may contain one or more transgenes and the genetic modification may take place prior to nuclear transfer and embryo reconstitution. Although micro-injection, analogous to injection into the male or female pronucleus of a zygote, may be used as a method of genetic modification, the invention is not limited to that methodology: mass transformation or transfection techniques can also be used e.g. electroporation, viral transfection or lipofection.

In the method of the invention described above, a diploid nucleus is transferred from a donor into the enucleated recipient oocyte. Donors which are diploid at the time of transfer are necessary in order to maintain the correct ploidy of the reconstituted embryo; therefore donors may be either in the $G_1$ phase or preferably, as is the subject of our co-pending UK patent application filed today, in the $G_0$ phase of the cell cycle.

The mitotic cell cycle has four distinct phases, G, S, $G_2$ and M. The beginning event in the cell cycle, called *start*, takes place in the $G_1$ phase and has a unique function. The decision or commitment to undergo another cell cycle is made at *start*. Once a cell has passed through *start*, it passes through the remainder of the $G_1$ phase, which is the pre-DNA synthesis phase. The second stage, the S phase, is when DNA synthesis takes place. This is followed by the $G_2$ phase, which is the period between DNA synthesis and mitosis. Mitosis itself occurs at the M phase. Quiescent cells (which include cells in which quiescence has been induced as well as those cells which are naturally quiescent, such as certain fully

differentiated cells) are generally regarded as not being in any of these four phases of the cycle; they are usually described as being in a $G_0$ state, so as to indicate that they would not normally progress through the cycle. The nuclei of quiescent $G_0$ cells, like the nuclei of $G_1$ cells, have a diploid DNA content; both of such diploid nuclei can be used in the present invention.

Subject to the above, it is believed that there is no significant limitation on the cells that can be used in nuclear donors: fully or partially differentiated cells or undifferentiated cells can be used as can cells which are cultured *in vitro* or abstracted *ex vivo*. The only limitation is that the donor cells have normal DNA content and be karyotypically normal. A preferred source of cells is disclosed in our co-pending UK patent application No. 9417831.6, a copy of which is annexed hereto and forms part of the disclosure of this application. It is believed that all such normal cells contain all of the genetic information required for the production of an adult animal. The present invention allows this information to be provided to the developing embryo by altering chromatin structure such that the genetic material can re-direct development.

Recipient cells useful in the invention care enucleated oocytes which are arrested in the metaphase of the second meiotic division. In most vertebrates, oocyte maturation proceeds *in vivo* to this fairly late stage of the egg maturation process and then arrests. At ovulation, the arrested oocyte is released from the ovary (and, if fertilisation occurs, the oocyte is naturally stimulated to complete meiosis). In the practice of the invention, oocytes can be matured either *in vitro* or *in vivo* and are

collected on appearance of the 1st polar body or as soon as possible after ovulation, respectively.

It is preferred that the recipient be enucleate. While it has been generally assumed that enucleation of recipient oocytes in nuclear transfer procedures is essential, there is no published experimental confirmation of this judgement. The original procedure described for ungulates involved splitting the cell into two halves, one of which was likely to be enucleated (Willadsen *Nature* **320** (6) 63-65 (1986)). This procedure has the disadvantage that the other unknown half will still have the metaphase apparatus and that the reduction in volume of the cytoplasm is believed to accelerate the pattern of differentiation of the new embryo (Eviskov *et al., Development* **109** 322-328 (1990)).

More recently, different procedures have been used in attempts to remove the chromosomes with a minimum of cytoplasm. Aspiration of the first polar body and neighbouring cytoplasm was found to remove the metaphase II apparatus in 67% of sheep oocytes (Smith & Wilmut *Biol. Reprod.* **40** 1027-1035 (1989)). Only with the use of DNA-specific fluorochrome (Hoechst 33342) was a method provided by which enucleation would be guaranteed with the minimum reduction in cytoplasmic volume (Tsunoda *et al., J. Reprod. Fertil.* **82** 173 (1988)). In livestock species, this is probably the method of routine use at present (Prather & First *J. Reprod. Fertil. Suppl.* **41** 125 (1990), Westhusin *et al., Biol. Reprod. (Suppl.)* **42** 176 (1990)).

There have been very few reports of non-invasive approaches to enucleation in mammals, whereas in

amphibians, irradiation with ultraviolet light is used as a routine procedure (Gurdon *Q. J. Microsc. Soc.* **101** 299-311 (1960)). There are no detailed reports of the use of this approach in mammals, although during the use of DNA-specific fluorochrome it was noted that exposure of mouse oocytes to ultraviolet light for more than 30 seconds reduced the developmental potential of the cell (Tsunoda *et al., J. Reprod. Fertil.* **82** 173 (1988)).

As described above enucleation may be achieved physically, by actual removal of the nucleus, pro-nuclei or metaphase plate (depending on the recipient cell), or functionally, such as by the application of ultraviolet radiation or another enucleating influence.

After enucleation, the donor nucleus is introduced either by fusion to donor cells under conditions which do not induce oocyte activation or by injection under non-activating conditions. In order to maintain the correct ploidy of the reconstructed embryo the donor nucleus must be diploid (i.e. in the $G_0$ or $G_1$ phase of the cell cycle) at the time of fusion.

Once suitable donor and recipient cells have been prepared, it is necessary for the nucleus of the former to be transferred to the latter. Most conveniently, nuclear transfer is effected by fusion. Activation should not take place at the time of fusion.

Three established methods which have been used to induce fusion are:

(1) exposure of cells to fusion-promoting chemicals, such as polyethylene glycol;

(2) the use of inactivated virus, such as Sendai virus; and

(3) the use of electrical stimulation.

Exposure of cells to fusion-promoting chemicals such as polyethylene glycol or other glycols is a routine procedure for the fusion of somatic cells, but it has not been widely used with embryos. As polyethylene glycol is toxic it is necessary to expose the cells for a minimum period and the need to be able to remove the chemical quickly may necessitate the removal of the zona pellucida (Kanka *et al.*, *Mol. Reprod. Dev.* **29** 110-116 (1991)). In experiments with mouse embryos, inactivated Sendai virus provides an efficient means for the fusion of cells from cleavage-stage embryos (Graham *Wistar Inst. Symp. Monogr.* **9** 19 (1969)), with the additional experimental advantage that activation is not induced. In ungulates, fusion is commonly achieved by the same electrical stimulation that is used to induce parthogenetic activation (Willadsen *Nature* **320** (6) 63-65 (1986), Prather *et al.*, *Biol. Reprod.* **37** 859-866 (1987)). In these species, Sendai virus induces fusion in a proportion of cases, but is not sufficiently reliable for routine application (Willadsen *Nature* **320** (6) 63-65 (1986)).

While cell-cell fusion is a preferred method of effecting nuclear transfer, it is not the only method that can be used. Other suitable techniques include microinjection (Ritchie and Campbell, *J. Reproduction and Fertility* Abstract Series No. 15, p60).

In a preferred embodiment of the invention, fusion of the oocyte karyoplast couplet is accomplished in the absence of activation by electropulsing in 0.3M mannitol solution

or 0.27M sucrose solution; alternatively the nucleus may be introduced by injection in a calcium free medium. The age of the oocytes at the time of fusion/injection and the absence of calcium ions from the fusion/injection medium prevent activation of the recipient oocyte.

In practice, it is best to enucleate and conduct the transfer s soon as possible after the oocyte reaches metaphase II. The time that this will be post onset of maturation (*in vitro*) or hormone treatment (*in vivo*) will depend on the species. For cattle or sheep, nuclear transfer should preferably take place within 24 hours; for pigs, within 48 hours; mice, within 12 hours; and rabbits within 20-24 hours. although transfer can take place later, it becomes progressively more difficult to achieve as the oocyte ages. High MPF activity is desirable.

Subsequently, the fused reconstructed embryo, which is generally returned to the maturation medium, is maintained without being activated so that the donor nucleus is exposed to the recipient cytoplasm for a period of time sufficient to allow the reconstructed embryo to become capable, eventually, of giving rise to a live birth (preferably of a fertile offspring).

The optimum period of time before activation varies from species to species and can readily be determined by experimentation. For cattle, a period of from 6 to 20 hours is appropriate. The time period should probably not be less than that which will allow chromosome formation, and it should not be so long either that the couplet activates spontaneously or, in extreme cases that it dies.

When it is time for activation, any conventional or other suitable activation protocol can be used. Recent experiments have shown that the requirements for parthogenetic activation are more complicated than had been imagined. It had been assumed that activation is an all-or-none phenomenon and that the large number of treatments able to induce formation of a pronucleus were all causing "activation". However, exposure of rabbit oocytes to repeated electrical pulses revealed that only selection of an appropriate series of pulses and control of the $Ca^{2+}$ was able to promote development of diploidized oocytes to mid-gestation (Ozil *Development* **109** 117-127 (1990)). During fertilization there are repeated, transient increases in intracellular calcium concentration (Cutbertson & Cobbold *Nature* **316** 541-542 (1985)) and electrical pulses are believed to cause analogous increases in calcium concentration. There is evidence that the pattern of calcium transients varies with species and it can be anticipated that the optimal pattern of electrical pulses will vary in a similar manner. The interval between pulses in the rabbit is approximately 4 minutes (Ozil *Development* **109** 117-127 (1990)), and in the mouse 10 to 20 minutes (Cutbertson & Cobbold *Nature* **316** 541-542 (1985)), while there are preliminary observations in the cow that the interval is approximately 20 to 30 minutes (Robl *et al.*, in *Symposium on Cloning Mammals by Nuclear Transplantation* (Seidel ed.), Colorado State University, 24-27 (1992)). In most published experiments activation was induced with a single electrical pulse, but new observations suggest that the proportion of reconstituted embryos that develop is increased by exposure to several pulses (Collas & Robl *Biol. Reprod.* **43** 877-884 (1990)). In any individual case, routine adjustments may be made to optimise the

number of pulses, the field strength and duration of the pulses and the calcium concentration of the medium.

In the practice of the invention, correct ploidy must be maintained during activation. It is desirable to inhibit microtubule polymerisation and prevent the production of multiple pronuclei, thereby to maintain correct ploidy. This can be achieved by the application of a microtubule inhibitor such as nocodazole at an effective concentration (such as about 5μg/ml). Colchecine and colcemid are other microtubule inhibitors.

The molecular component of microtubules (tubulin) is in a state of dynamic equilibrium between the polymerised and non-polymerised states. Microtubule inhibitors such as nocodazole prevent the addition of tubulin molecules to microtubules, thereby disturbing the equilibrium and leading to microtubule depolymerisation. It is preferred to add the microtubule inhibitor a sufficient time before activation to ensure complete, or almost complete, depolymerisation of the microtubules. Twenty to thirty minutes is likely to be sufficient in most cases.

The microtubule inhibitor should remain present after activation until pronuclei formation. It should be removed thereafter, and in any event before the first division takes place.

In a preferred embodiment of the invention at 30-42 hours post onset of maturation (bovine and ovine, i.e. 6-18 hours post nuclear transfer) the reconstructed oocytes are placed into medium containing nocodazole (5μg/ml) and activated using conventional protocols. Incubation in nocodazole may be continued for 4-6 hours following the

activation stimulus (dependent upon species and oocyte age).

According to a second aspect of the invention, there is provided a viable reconstituted animal embryo prepared by a method as described previously.

According to a third aspect of the invention, there is provided a method of preparing an animal, the method comprising:

(a) reconstituting an animal embryo as described above; and
(b) causing an animal to develop to term from the embryo; and
(c) optionally, breeding from the animal so formed.

Step (a) has been described in depth above.

The second step, step (b) in the method of this aspect of the invention is to cause an animal to develop to term from the embryo. This may be done directly or indirectly. In direct development, the reconstituted embryo from step (a) is simply allowed to develop without further intervention beyond any that may be necessary to allow the development to take place. In indirect development, however, the embryo may be further manipulated before full development takes place. For example, the embryo may be split and the cells clonally expanded, for the purpose of improving yield.

Alternatively or additionally, it may be possible for increased yields of viable embryos to be achieved by means of the present invention by clonal expansion of

donors and/or if use is made of the process of serial (nuclear) transfer. A limitation in the presently achieved rate of blastocyst formation may be due to the fact that a majority of the embryos do not "reprogram" (although an acceptable number do). If this is the case, then the rate may be enhanced as follows. Each embryo that does develop itself can be used as a nuclear donor at the 32-64 cell stage; alternatively, inner cell mass cells can be used at the blastocyst stage. If these embryos do indeed reflect those which have reprogrammed gene expression and those nuclei are in fact reprogrammed (as seems likely), then each developing embryo may be multiplied in this way by the efficiency of the nuclear transfer process. The degree of enhancement likely to be achieved depends upon the cell type. In sheep, it is readily possible to obtain 55% blastocyst stage embryos by transfer of a single blastomere from a 16 cell embryo to a preactivated "Universal Recipient" oocyte. So it is reasonable to hypothesise that each embryo developed from a single cell could give rise to eight at the 16 cell stage. Although these figures are just a rough guide, it is clear that at later developmental stages the extent of benefit would depend on the efficiency of the process at that stage.

Aside from the issue of yield-improving expediencies, the reconstituted embryo may be cultured, *in vivo* or *in vitro* to blastocyst.

Experience suggests that embryos derived by nuclear transfer are different from normal embryos and sometimes benefit from or even require culture conditions *in vivo* other than those in which embryos are usually cultured (at least *in vivo*). The reason for this is not known.

In routine multiplication of bovine embryos, reconstituted embryos (many of them at once) have been cultured in sheep oviducts for 5 to 6 days (as described by Willadsen, In Mammalian Egg Transfer (Adams, E.E., ed.) 185 CRC Press, Boca Raton, Florida (1982)). In the practice of the present invention, though, in order to protect the embryo it should preferably be embedded in a protective medium such as agar before transfer and then dissected from the agar after recovery from the temporary recipient. The function of the protective agar or other medium is twofold: first, it acts as a structural aid for the embryo by holding the zona pellucida together; and secondly it acts as barrier to cells of the recipient animal's immune system. Although this approach increases the proportion of embryos that form blastocysts, there is the disadvantage that a number of embryos may be lost.

If *in vitro* conditions are used, those routinely employed in the art are quite acceptable.

At the blastocyst stage, the embryo may be screened for suitability for development to term. Typically, this will be done where the embryo is transgenic and screening and selection for stable integrants has been carried out. Screening for non-transgenic genetic markers may also be carried out at this stage. However, because the method of the invention allows for screening of donors at an earlier stage, that will generally be preferred.

After screening, if screening has taken place, the blastocyst embryo is allowed to develop to term. This will generally be *in vivo*. If development up to blastocyst has taken place *in vitro*, then transfer into the final recipient animal takes place at this stage. If

blastocyst development has taken place *in vivo*, although in principle the blastocyst can be allowed to develop to term in the pre-blastocyst host, in practice the blastocyst will usually be removed from the (temporary) pre-blastocyst recipient and, after dissection from the protective medium, will be transferred to the (permanent) post-blastocyst recipient.

In optional step (c) of this aspect of the invention, animals may be bred from the animal prepared by the preceding steps. In this way, an animal may be used to establish a herd or flock of animals having the desired genetic characteristic(s).

Animals produced by transfer of nuclei from a source of genetically identical cells share the same nucleus, but are not strictly identical as they are derived from different oocytes. The significance of this different origin is not clear, but may affect commercial traits. Recent analyses of the mitochondrial DNA of dairy cattle in the Iowa State University Breeding Herd revealed associated with milk and reproductive performance (Freeman & Beitz, In Symposium on Cloning Mammals by Nuclear Transplantation (Seidel, G. E. Jr., ed.) 17-20, Colorado State University, Colorado (1992)). It remains to be confirmed that similar effects are present throughout the cattle population and to consider whether it is possible or necessary in specific situations to consider the selection of oocytes. In the area of cattle breeding the ability to produce large numbers of embryos from donors of high genetic merit may have considerable potential value in disseminating genetic improvement through the national herd. The scale of application will depend upon the cost of each embryo and the proportion of transferred embryos able to develop to term.

By way of illustration and summary, the following scheme sets out a typical process by which transgenic and non-transgenic animals may be prepared. The process can be regarded as involving five steps:

(1) isolation of diploid donor cells;
(2) optionally, transgenesis, for example by transfection with suitable constructs, with or without selectable markers;
(2a) optionally screen and select for stable integrants - skip for micro-injection;
(3) embryo reconstitution by nuclear transfer;
(4) culture, *in vivo* or *in vitro*, to blastocyst;
(4a) optionally screen and select for stable integrants - omit if done at 2a - or other desired characteristics;
(5) transfer if necessary to final recipient.

This protocol has a number of advantages over previously published methods of nuclear transfer:

1) The chromatin of the donor nucleus can be exposed to the meiotic cytoplasm of the recipient oocyte in the absence of activation for appropriate periods of time. This may increase the "reprogramming" of the donor nucleus by altering the chromatin structure.

2) Correct ploidy of the reconstructed embryo is maintained when $G_0/G_1$ nuclei are transferred.

3) Previous studies have shown that activation responsiveness of bovine/ovine oocytes increases with age. One problem which has previously been observed is that in unenucleated aged oocytes duplication of the

meiotic spindle pole bodies occurs and multipolar spindles are observed. However, we report that in embryos reconstructed and maintained with high MPF levels although nuclear envelope breakdown and chromatin condensation occur no organised spindle is observed. The prematurely condensed chromosomes remain in a tight bunch, therefore we can take advantage of the ageing process and increase the activation response of the reconstructed oocyte without adversely affecting the ploidy of the reconstructed embryo.

According to a fourth aspect of the invention, there is provided an animal prepared as described above.

Preferred features of each aspect of the invention are as for each other aspect, *mutatis mutandis*.

The invention will now be described by reference to the accompanying Examples which are provided for the purposes of illustration and are not to be construed as being limiting on the present invention. In the following description, reference is made to the accompanying drawing, in which:

FIGURE 1 shows the rate of maturation of bovine oocytes *in vitro*.

Example 1: "MAGIC" Procedure using Bovine Oocytes

Recipient oocytes the subject of this experimental procedure are designated MAGIC (Metaphase Arrested $G_1/G_0$ AcceptIng Cytoplast) Recipients.

The nuclear and cytoplasmic events during *in vitro* oocyte maturation were studied. In addition the roles of fusion and activation in embryos reconstructed at different ages were also investigated. The studies have shown that oocyte maturation is asynchronous; however, a population of matured oocytes can be morphologically selected at 18 hours (Figure 1).

In Figure 1 ovaries were obtained from a local abattoir and maintained at 28-32°C during transport to the laboratory. Cumulus oocyte complexes (COC's) were aspirated from follicles 3-10mm in diameter using a hypodermic needle (1.2mm internal diameter) and placed into sterile plastic universal containers. The universal containers were placed into a warmed chamber (35°C) and the follicular material allowed to settle for 10-15 minutes before pouring off three quarters of the supernatant. The remaining follicular material was diluted with an equal volume of dissection medium (TCM 199 with Earles salts (Gibco), 75.0 mg/l kanamycin, 30.0mM Hepes, pH 7.4, osmolarity 280 mOsmols/Kg $H_2O$) supplemented with 10% bovine serum, transferred into an 85mm petri dish and searched for COC's under a dissecting microscope. Complexes with at least 2-3 compact layers of cumulus cells were selected washed three times in dissection medium and transferred into maturation medium (TC medium 199 with Earles salts (Gibco), 75mg/l kanamycin, 30.0mM Hepes, 7.69mM $NaHCO_3$, pH 7.8, osmolarity 280 mOsmols/Kg $H_2O$) supplemented with 10% bovine serum and $1x10^6$ granulosa cells/ml and cultured on a rocking table at 39°C in an atmosphere of 5% $CO_2$ in air. Oocytes were removed from the maturation dish and wet mounted on ethanol cleaned glass slides under coverslips which were attached using a mixture of 5% petroleum jelly 95% wax.

Mounted embryos were then fixed for 24 hours in freshly prepared methanol: glacial acetic acid (3:1), stained with 45% aceto-orcein (Sigma) and examined by phase contrast and DIC microscopy using a Nikon Microphot-SA, the graph in Figure 1 shows the percentage of oocytes at MII and those with a visible polar body.

If maturation is then continued until 24 hours these oocytes activate at a very low rate (24%) in mannitol containing calcium (Table 1a). However, removal of calcium and magnesium from the electropulsing medium prevents any activation.

Table 1a shows activation of bovine follicular oocytes matured *in vitro* for different periods. Oocytes were removed from the maturation medium, washed once in activation medium, placed into the activation chamber and given a single electrical pulse of 1.25kV/cm 80$\mu$s.

Table 1a

| N | AGE (HRS) | % ACTIVATION |
|---|---|---|
| 73 | 24 | 24.6 |
| 99 | 30 | 84.8 |
| 55 | 45 | 92.7* |

*many 2 or more pronuclei

Table 1b shows activation response of *in vitro* matured bovine oocytes sham enucleated at approximately 22 hours post onset of maturation. Oocytes were treated exactly as for enucleation, a small volume of cytoplasm was aspirated not containing the metaphase plate. After manipulation the oocytes were given a single DC pulse of

1.25 KV/cm and returned to the maturation medium, at 30 hpm and 42 hpm groups of oocytes were mounted, fixed and stained with aceto-orcein. The results show the number of oocytes at each time point from five individual experiments.

Table 1b

| EXPERIMENT | 30 hpm | 42 hpm |
|---|---|---|
| 1 | 1/8 | - |
| 2 | 0/24 | 0/30 |
| 3 | 0/21 | 0/22 |
| 4 | 0/27 | 0/25 |
| 5 | 0/19 | 0/19 |

The results also show that:

i) these oocytes can be enucleated at 18 hours post onset of maturation;

ii) enucleated oocytes can be fused to donor blastomeres/cells in either 0.3M mannitol or 0.27M sucrose alternatively the donor the cells or nuclei can be injected in calcium free medium in the absence of any activation response;

iii) reconstructed embryos or enucleated pulsed oocytes can be cultured in maturation medium and do not undergo spontaneous activation;

iv) the transferred nucleus is seen to undergo nuclear envelope breakdown (NEBD) and chromosome condensation. No organised meiotic/mitotic spindle is observed

regardless of the cell cycle stage of the transferred nucleus;

v) such manipulated couplets will activate at 30 hours and 42 hours with a frequency equal to unmanipulated control oocytes;

vi) no polar body is observed following subsequent activation, regardless of the cell cycle stage of the transferred nucleus;

viii) upon subsequent activation 1-5 micronuclei are formed per reconstructed zygote (Table 2).

Table 2 shows pronuclear formation in enucleated oocytes fused to primary bovine fibroblasts (24 hpm) and subsequently activated (42hpm). The results represent five separate experiments. Oocytes were divided into two groups, group A were incubated in nocodazole for 1 hour prior to activation and for 6 hours following activation. Group B were not treated with nocodazole. Activated oocytes were fixed and stained with aceto-orcein 12 hours post activation. The number of pronuclei (PN) in each parthenote was then scored under phase contrast. The results are expressed as the percentage of activated oocytes containing 1 or more pronuclei.

Table 2

| | TOTAL | 1 PN | 2 PN | 3 PN | 4 PN | >4 PN |
|---|---|---|---|---|---|---|
| GROUP A | 52 | 100 | 0 | 0 | 0 | 0 |
| GROUP B | 33 | 45.2 | 25.8 | 16.1 | 3.2 | 9.7 |

The absence of an organised spindle and the absence of a polar body suggests that in order to maintain ploidy in the reconstructed embryo then only a diploid i.e. $G_0/G_1$ nucleus should be transferred into this cytoplasmic situation. Incubation of activated oocytes in the presence of the microtubule inhibitor nocodazole for 5 hours, 1 hour prior to and following the activation stimulus prevents the formation of micronuclei (Table 2) and thus when the donor nucleus is in the $G_0/G_1$ phase of the cell cycle the correct ploidy of the reconstructed embryo is maintained.

Results

In preliminary experiments this technique has been applied to the reconstruction of bovine embryos using primary fibroblasts synchronised in the $G_0$ phase of the cell cycle by serum starvation for five days. The results are summarised in Table 3.

Table 3 shows development of bovine embryos reconstructed by nuclear transfer of serum starved ($G_0$) bovine primary fibroblasts into enucleated unactivated MII oocytes. Embryos were reconstructed at 24 hpm and the fused couplets activated at 42 hpm. Fused couplets were incubated in nocodazole (5$\mu$g/ml) in M2 medium for 1 hour prior to activation and 5 hours post activation. Couplets were activated with a single DC pulse of 1.25 KV/cm for 80$\mu$sec.

Table 3

| EXPERIMENT NUMBER | NUMBER OF BLASTOCYSTS/ TOTAL NUMBER OF FUSED COUPLETS | % BLASTOCYSTS |
|---|---|---|
| 1 | 1/30 | 3.3 |
| 2 | 4/31 | 12.9 |

Example 2: "MAGIC" Procedure using Ovine Oocytes

Similar observations to those in Example 1 have also been made in ovine oocytes which have been matured *in vivo;*

Freshly ovulated oocytes can be retrieved by flushing from the oviducts of superstimulated ewes 24 hours after prostaglandin treatment. The use of calcium magnesium free PBS/1.0% FCS as a flushing medium prevents oocyte activation. Oocytes can be enucleated in calcium free medium and donor cells introduced as above in the absence of activation. No organised spindle is observed, multiple nuclei are formed upon subsequent activation and this may be suppressed by nocodazole treatment.

Results

In preliminary experiments in sheep, a single pregnancy has resulted in the birth of a single live lamb. The results are summarised in Tables 4 and 5.

Table 4 shows development of ovine embryos reconstructed by transfer of an embryo derived established cell line to unactivated enucleated *in vivo* matured ovine oocytes. Oocytes were obtained from superstimulated Scottish blackface ewes, the cell line was established from the

embryonic disc of a day 9 embryo obtained from a Welsh mountain ewe. Reconstructed embryos were cultured in the ligated oviduct of a temporary recipient ewe for 6 days, recovered and assessed for development.

Table 4

| DATE OF NUCLEAR TRANSFER | PASSAGE NUMBER | NUMBER OF MORULA, BLA STOCSTS/ TOTAL NUMBER |
|---|---|---|
| | | |
| 17.1.95 | 6 | 4/28 |
| 19.1.95 | 7 | 1/10 |
| 31.1.95 | 13 | 0/2 |
| 2.2.95 | 13 | 0/14 |
| 7.2.95 | 11 | 1/9 |
| 9.2.95 | 11 | 1/2 |
| 14.2.95 | 12 | |
| 16.2.95 | 13 | 3/13 |
| | | |
| TOTAL | | 10/78 (12.8%) |

Table 5 shows induction of pregnancy following transfer of all morula/blastocyst stage reconstructed embryos to the uterine horn of synchronised final recipient blackface ewes. The table shows the total number of embryos from each group transferred the frequency of pregnancy in terms of ewes and embryos, in the majority of cases 2 embryos were transferred to each ewe. A single twin pregnancy was established which resulted in the birth of a single live lamb.

Table 5

| PASSAGE NUMBER | "MAGIC" |
|---|---|
| P6 | 4 |
| P7 | 1 |
| P11 | 2 |
| P12 | 0 |
| P13 | 3 |
| | |
| TOTAL MOR/BL | 10 |
| TOTAL NUMBER EWES | 6 |
| PREGNANT EWES % | 1 (16.7) |
| FOETUSES/ TOTAL TRANSFERRED (%) | 2/10 (20.0) |

CLAIMS

1. A method of reconstituting an animal embryo, the process comprising transferring a diploid nucleus into an oocyte which is arrested in the metaphase of the second meiotic division without concomitantly activating the oocyte, keeping the nucleus exposed to the cytoplasm of the recipient for a period of time sufficient for the embryo to become capable of giving rise to a live birth and subsequently activating the reconstituted embryo while maintaining correct ploidy.

2. A method as claimed in claim 1, in which the animal is an ungulate species.

3. A method as claimed in claim 2, in which the animal is a cow or bull, pig, goat, sheep, camel or water buffalo.

4. A method as claimed in any one of claims 1 to 3, in which the donor nucleus is genetically modified.

5. A method as claimed in any one of claims 1 to 4, wherein the diploid nucleus is donated by a quiescent cell.

6. A method as claimed in any one of claims 1 to 5, wherein the recipient oocyte is enucleate.

7. A method as claimed in any one of claims 1 to 6, wherein nuclear transfer is achieved by cell fusion.

8. A method as claimed in any one of claims 1 to 7, wherein the animal is a cow or bull and wherein the donor

nucleus is kept exposed to the recipient cytoplasm for a period of from 6 to 20 hours prior to activation.

9. A method as claimed in any one of claims 1 to 8, wherein correct ploidy is maintained during activation by microtubule inhibition.

10. A method as claimed in claim 9, wherein microtubule inhibition is achieved by the application of nocodazole.

11. A method of preparing an animal, the method comprising:

(a) reconstituting an animal embryo as claimed in any preceding claim;
(b) causing an animal to develop to term from the embryo; and
(c) optionally, breeding from the animal so formed.

12. A method as claimed in claim 11, wherein the animal embryo is further manipulated prior to full development of the embryo.

13. A method as claimed in claim 12, wherein more than one animal is derived from the embryo.

14. A reconstituted animal embryo which is capable of giving rise to a live birth and is prepared by a method as claimed in any one of claims 1 to 10.

15. An animal prepared by a method as claimed in any one of claims 11 to 13.

16. An animal developed from an embryo as claimed in claim 14.

## ABSTRACT

### BIOLOGICAL MANIPULATION

A method of reconstituting an animal embryo involves transferring a diploid nucleus into an oocyte which is arrested in the metaphase of the second meiotic division. The oocyte is not activated at the time of transfer, so that the donor nucleus is kept exposed to the recipient cytoplasm for a period of time. The diploid nucleus can be donated by a cell in either the $G_0$ or $G_1$ phase of the cell cycle at the time of transfer. Subsequently, the reconstituted embryo is activated. Correct ploidy is maintained during activation, for example, by incubating the reconstituted embryo in the presence of a microtubule inhibitor such as nocodazole. The reconstituted embryo may then give rise to one or more live animal births. The invention is useful in the production of transgenic animals as well as non-transgenics of high genetic merit.

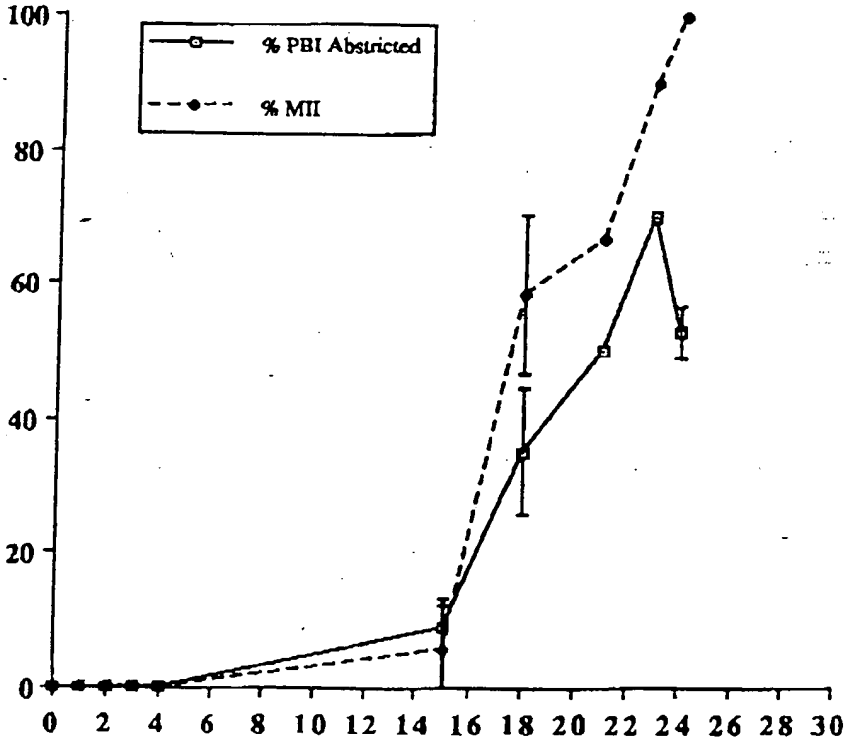
% PBI Abstricted
% MII
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
MATURATION TIME HOURS

FIG. 1.

ANNEX

# BIOLOGICAL MANIPULATION

This invention relates to the generation of animals, including but not being limited to transgenic animals, and to cells useful in their generation.

The cloning and propagation of cells capable of developing into healthy animals are objectives which have been sought for some time by animal breeders and by producers of transgenic animals. Animal breeders dealing with non-transgenic animals have long sought a means of cloning animals of high genetic merit. The nature of such merit will of course depend on the objectives of the breeder, but it is clear that the dairy industry, to take one example, would benefit from the ability to limit births of calves to those of a single sex.

Gene transfer (transgenesis) has been widely used in the mouse to address questions of gene function, and more sparingly in domestic species, in attempts to alter characters with high economic value. Currently, whole animal transgenesis in species other than mouse can only be achieved by pronuclear injection or, less commonly, by viral transfection. A method from transgenic cultured cells in the mouse is also available, known as the embryonic stem cell (ES cell) system. This system depends upon the isolation in culture of a specific embryonic lineage which may be modified *in vitro* while retaining its unique ability to participate in development following transplantation as an intact cell, to early embryos. Proven ES cells are not available in species other than mouse and ES cell nuclei do not support mouse development when transferred to enucleated zygotes or oocytes using present procedures.

ANNEX

Although a number of abstracts over the years have reported the establishment of lines of pluripotent embryonic cells derived from morula or blastocyst stage embryos of ungulates, two of the most recent being Stice *et al.*, *Theriogenology* **41** 301 (1994) and Strelchenko & Stice, *Theriogenology* **41** 304 (1994), there is as yet no evidence that permanent ES-like cell lines in ungulates can give rise to healthy animals.

Pronuclear microinjection is currently the only practicable procedure for gene transfer in ungulates, particularly farm animals. The efficiency of transgenesis in these species suffers due to the granular nature of the cytoplasm which renders it difficult to visualise pronuclei and because of the smaller number of eggs which may be obtained per animal. Additional constraints are the huge cost of animals and the need, in cattle, to transfer single embryos to each recipient female in order to avoid freemartins.

In contrast to gene transfer, which is much easier in mice than in farm animals, nuclear transfer (NT) in farm animals has been relatively more successful than in the mouse. Calves and lambs have been generated using nuclei from cells of the blastocyst inner cell mass in both cattle (Keefer *et al.*, *Biol. Reprod.* **50** 935-939 (1994) and Sims & First, *Proc. Nat'l. Acad. Sci. USA* **90** 6143-6147 (1994)) and sheep (Smith & Wilmut, *Biol. Reprod.* **40** 1027-1035 (1989)). Similar experiments in mice are controversial, but have probably not yielded pregnancies.

Embryonic stem cells (ES cells) are tissue culture cells isolated from the inner cell mass of the mouse blastocyst which retain in culture their ability to participate in

normal development. This capacity is dramatically demonstrated when ES cells are returned to the early embryonic environment, wherein they participate in normal development, giving rise to chimeric animals whose tissues are a mosaic of host embryo and ES cell genotypes. Where ES cells contribute to primordial germ cells, then genetic manipulations to ES cells *in vitro* can be passed on to transgenic animals. There has been much interest in the isolation of ES cells from farm animals due, largely, to the expectation that ES nuclei might be competent for nuclear transfer, offering a more efficient route to transgenesis. There is, however, no evidence that competence for NT is a property of ES cells.

ES cells are defined by their ability to make germline chimeras. In the development of transgenic farm animals, because of the opportunities for pre-transfer screening and gene targeting and to avoid an extra chimeric generation, there is more interest in achieving transgenesis through nuclear transfer from cultured cells. The present invention, therefore, arises from the idea that an efficient gene transfer system through NT from cultured cells may be achieved without the necessity of isolating ungulate ES lines.

Sims & First (*Proc. Nat'l. Acad. Sci. USA* **90** 6143-6147 (1994)) describe the production of calves by transfer of nuclei from cultured inner cell mass (ICM) of bovine blastocysts. However, based on the data given in this paper, there is no evidence of the establishment of a true cell line from the ICM cells. For example, the authors state:

> ICM cells from day 9 and 10 bovine blastocysts multiplied in culture when culture in CR1aa

ANNEX

> plus SIT and 5% FCS with some lines reaching 2,000 cells after 2 weeks of culture. [page 6144, right hand column, lines 40-43]

2,000 is a remarkably low number of cells for anything that could reasonably be called a cell line. A single confluent 25 cm flask of ES cells, for example, would generally contain $10^6$ to $10^7$ cells, and a line would grow to $10^9$ to $10^{10}$ cells after five or six passages.

In fact, it is clear that Sims & First do not use conventional passaging techniques: instead of attaching cells to a tissue culture substrate and detaching and replating the cells when they reach confluence, as is traditional, the authors maintained the ICM cells in suspension culture for up to two months. If no more than 2,000 cells resulted from this process, little cell division can have been taking place.

It would be desirable to establish a genuine cell line of cells which are totipotent for nuclear transfer. The present invention achieves that goal and is based on the discovery that cells derived from the embryonic disc of blastodermic vesicles can be used to establish such lines.

According to a first aspect of the present invention there is provided an animal cell line derived from an embryonic disc of an ungulate blastodermic vesicle, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, cells of which cell line are totipotent for nuclear transfer.

Such cells, designated in this specification as TNT cells, can be isolated, individually or collectively, and themselves form part of the invention, according to a

second aspect of which there are provided isolated animal cells which are derived from an embryonic disc of an ungulate blastodermic vesicle, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, and which are totipotent for nuclear transfer.

Stages of non-ungulate embryonic development which are equivalent to the blastodermic vesicle in ungulates are those at or immediately after the determination of the three germ layers at gastrulation; examples include the early egg cylinder stage in rodents and the early gastrula in avian species. Tissues from these stages which are equivalent to the ungulate embryonic disc are those which exclude extra-embryonic lineages; examples include embryonic ectoderm plus visceral endoderm in rodents and embryonic disc in avian species. Where, in the relevant species, embryonic ectoderm can be dissected free of endoderm, embryonic ectoderm alone is the preferred tissue.

Totipotentiality is the capacity of a cell to differentiate into all cell types; it is a property of both nucleus and cytoplasm. In this specification, a cell is said to be "totipotent for nuclear transfer" or "TNT" if, following nuclear transfer from that cell to an oocyte, a healthy animal develops to term. Nuclear transfer may be achieved by fusion of the TNT cell with an oocyte, zygote or early (for example, two cell) blastomere. In the case of the present invention, totipotentiality is a property of the TNT nucleus and the recipient cell cytoplasm. It is to be emphasised, though, that no special properties of the recipient cell are required: any normal (generally enucleated) oocyte, zygote or early blastomere will suffice. Enucleation may

be achieved physically, by actual removal of the nucleus, pro-nuclei or metaphase plate (depending on the recipient cell), or functionally, such as by the application of ultraviolet radiation or another enucleating influence.

Cell lines of the invention can be passaged by conventional means and can be kept in permanent culture. By "permanent" culture is meant culture in which significant reproduction of the cells take place and which can be propagated by passaging; the culture is not necessarily kept indefinitely, but can certainly survive for more than ten passages, by which time it would be regarded as permanently established by those skilled in the tissue culture methodology. At that stage, approximately $10^9$ to $10^{10}$ or more cells may be present in the culture.

In principle, the invention is applicable to all animals, including birds such as domestic fowls. In practice, however, it will be to (non-human) mammals, particularly placental mammals, that the greatest commercially useful applicability is presently envisaged. TNT cells and cell lines derived from small mammals such as rabbits and rodents, especially mice and rats, may be useful in some applications, but it is with ungulates, particularly economically important ungulates such as cattle, sheep, goats, water buffalo, camels and pigs that the invention is likely to be most useful, both as a means for cloning animals and as a means for generating transgenic animals.

Isolation of TNT cells from embryos arising from selected matings among elite stock could be used to clone animals of one or more desired genetic characteristics or high genetic merit generally. This would include the

capability of limiting births to a single sex, which as previously mentioned is of particular importance in the dairy industry.

For the generation of transgenic animals, TNT cells can be genetically manipulated. Then, by a process of nuclear transfer, which in itself is known (see, for example, Campbell *et al.*, *Biol. Reprod.* **50** 1385-1393 (1994)), transgenic animals may be produced from the genetically altered, cultured cells. The overall procedure is expected to have several advantages -- particularly in the generation of transgenic farm animals -- over conventional procedures for generating transgenics, namely (1) that fewer recipient animals will be required, (2) that multiple syngeneic founders may be generated using clonal TNT cells, and (3) that the system will permit subtle genetic alteration by gene targeting.

It should be noted that the term "transgenic", in relation to animals, should not be taken to be limited to referring to animals containing in their germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted, duplicated, activated or modified is a transgenic animal for the purposes of this invention as much as an animal to whose germline an exogenous DNA sequence has been added.

According to a third aspect of the invention, there is provided a process for the preparation of an animal, the

process comprising reconstituting an animal embryo by nuclear transfer from a TNT cell as described above, allowing the embryo to develop to term and optionally breeding from the animal so formed.

In embodiments of this aspect of the invention in which the animal is transgenic, TNT cells may be genetically modified prior to nuclear transfer. Although micro-injection, analogous to injection into the male or female pronucleus of a zygote, may be used as a method of genetic modification, the invention is not limited to that methodology: mass transformation or transfection techniques can also be used.

By way of illustration, the following scheme sets out a typical process by which transgenic animals may be prepared. The process can be regarded as involving five steps:

(1) isolation of TNT cells;
(2) transgenesis, for example by transfection with suitable constructs, with or without selectable markers;
(2a) optionally screen and select for stable integrants - skip for microinjection;
(3) embryo reconstitution by nuclear transfer;
(4) culture, *in vivo* or *in vitro*, to blastocyst;
(4a) optionally screen and select for stable integrants - omit if done at 2a;
(5) transfer to final recipient.

**Isolation of TNT cells**

TNT cells can be isolated from explants of the embryonic disc of animals. More particularly, TNT cells can be

isolated from explants of the embryonic discs of early blastodermic vesicles, for example at days 9 and 10 in sheep, and at equivalent stages in other animals. For cattle and pigs, the equivalent stage would be days 11 and 12 of the blastodermic vesicle, and in rodents the equivalent would be days 5 and 6 of the egg cylinder.

The most successful procedure for the isolation of TNT cells, which itself forms a fourth aspect of the invention, comprises explanting at least part of the embryonic disc of an animal embryo at the blastodermic vesicle stage in the case of an ungulate, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, allowing the development of an ES-like colony of undifferentiated cells until they have acquired an enlarged, more epithelial phenotype (which will usually be by about passage 2) and entering a cell or cells from such a colony into reproductive culture until required.

In practice, substantially the whole of the embryonic disc, essentially free of trophectoderm, will usually be taken, for example by microdissection or immunosurgery, from the embryo. Explantation will be onto a suitable tissue culture substrate or medium, such as a primary mouse fibroblast feeder layer (or other inactivated feeder) layer in ES medium, or with conditioned or supplemented medium. Approximately 10% of explants have in practice been found to give rise to the ES-like colonies of undifferentiated cells, which by passage 2 have acquire the enlarged, more epithelial phenotype referred to above. Such a colony, or at least one or more of the cells from it, may be entered into permanent culture, as shown for example in Example 1 below. TNT

cells from the sheep at passage 3 support development of lambs to term following nuclear transfer to enucleated oocytes (see Example 2 below).

The TNT strategy differs from the ES strategy in that cell lines are isolated from later stage blastodermic vesicles (days 9 and 10 in sheep) and are not required to generate chimeras. TNT cells do not display the classic ES phenotype of small, rounded, undifferentiated cells and are more epithelial in character, growing as a flat monolayer.

The TNT strategy of the present invention also differs from that of Sims & First (*Proc. Nat'l. Acad. Sci. USA* **90** 6143-6147 (1994)) in that they isolated cells at an earlier stage of development from the inner cell mass of the blastocyst, rather than the embryonic disc of the blastodermic vesicle, and did not appear to have established true cell lines.

**Transfection and nuclear transfer**

It should be possible to introduce transgene constructs, which may be selectable, by a variety of methods. This is an advantage over the technique of Sims & First: the absence of true cell lines in their paper indicates that their technique will probably be restricted to microinjection; this does not represent much of an improvement over existing pronuclear microinjection techniques. Since the present invention enables the establishment of a line of rapidly dividing cells in culture, mass transformation or transfection techniques can be used, including electroporation, viral-mediated transfection and lipofection. Further, since so many cells are present, the present invention enables

investigators to capitalise on events which happen at low frequency: an example of a low frequency event is homologous recombination, which can be harnessed for gene targeting.

If a selectable genetic construct is used, the transfection or transformation step can be followed by culture in selective medium. The invention is not limited to the use of any particular technique for the introduction of a transgene: the foregoing examples are given merely by way of illustration. Using microinjection, it should be possible to introduce transgenes at passage 2 or 3 without selection. Injected DNA integrates stably at a frequency of about 20% in cultured cells (Lovell-Badge, "Introduction of DNA into embryonic stem cells" In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. IRL Press, Oxford, E. J. Robertson, ed. pp 153-182, 1987). Therefore, on the assumption that the transgene has no direct effect upon viability, 20% of embryos reconstituted from unselected, injected TNT cells and cultured to blastocyst will be stably transgenic. The numbers of final recipient animals required may be further reduced by employing biopsy procedures prior to final transfer (step 4a above), although there will be a concomitant reduction in viability.

**Culture to blastocyst and final embryo transfer**

The average proportion of injected and transferred pronuclear ungulate eggs which generate transgenics is below 1.0% (Clark *et al*, "Germline Manipulation: Applications in Agriculture and Biotechnology" In: *Transgenic Animals*. Academic Press, London F. Grosveld and G. Kollias, eds. pp 247-270 (1992)). This means that

for a single cattle transgenic project, several hundred embryo transfers are required, involving similar numbers of recipient females. Hence several hundred cattle need to be maintained for a period of 15 months (embryo transfer to weaning). With TNT cells, preselection of transgenic clones would mean that 100% of liveborn animals would be transgenic, and the only losses would be due to the proportion of TNT-derived blastocysts transferred to final recipients which fail to develop to term. Using *in vivo* culture in temporary recipients, preliminary data suggest that development to blastocyst strongly correlates with development to term (Table 1), raising the possibility that the numbers of recipients required can be reduced by an order of magnitude using the TNT system. These advantages can be gained with quite early passage TNT cells (passages 4-6 depending upon transfection method).

**Table 1. Fusion, development to morulae/blastocyst and development to term of reconstituted embryos from TNT and 16 cell blastomere nuclei**

| Cell Line | Passage | Fused | Number transferred | Lambs |
|---|---|---|---|---|
| TNT/2 | P1 | 15 | 2 | 0 |
| TNT/3 | P1 | 16 | 2 | 0 |
| TNT/4 | P2 | 23 | 1 | stillborn |
| TNT/4 | P3 | 49 | 2 | 2 |
| TNT subtotal | | 103 | 7 | 2 |
| 16 Cell | | 72 | 9 | 4 |

**Gene targeting**

Sheep TNT cells at passage 3 and beyond grow rapidly in culture, with a doubling time of approximately 24 hours. Gene targeting regimes similar to those used for ES cells require $10^7$ cells for the initial electroporation, although in principle this can be reduced. These numbers

of TNT cells are available by passage 4 (approximately 23 cell divisions). Development to term from TNT cells at passage 3 has already been achieved. Subsequent selection will involve a further 14-20 cell divisions (equivalent to passage 7-9). It is anticipated that if TNT nuclei at later passages (for example, passage 10) are still able to support development to term, then it will become possible to do gene targeting in ungulates. Procedures using cell surface markers followed by cell sorting could even reduce the passage number to 5 or 6.

Preferred features for each aspect of the invention are as for each other aspect, *mutatis mutandis*.

The invention will now be illustrated by the following examples. The examples refer to the drawings, in which:

FIGURE 1 shows the appearance of TNT/4 cells at passage 4 (circled) growing on an inactivated fibroblast feeder layer (arrowed). TNT cells have an enlarged, flattened, epithelial phenotype, contrasting sharply with ES cells, which are small, rounded and have a high nucleus:cytoplasm ratio; and

FIGURE 2 shows a lamb (left) derived from fusion of a Welsh Mountain-derived TNT cell to a Scottish Blackface oocyte, followed by transfer to a Scottish Blackface foster mother (right). Lamb and foster mother show characteristic markings of Welsh Mountain (TNT/4 genotype) and Scottish Blackface (recipient oocyte genotype) respectively.

**EXAMPLE 1: Isolation of Sheep TNT Cell Lines**

The oestrus cycles of Welsh Mountain ewes were

synchronised with progesterone impregnated sponges. Ewes were then superovulated with 2 daily injections of equine follicle-stimulating hormone. Ovulation was induced with a single injection of gonadotropin release hormone (gnRH) and animals were artificially inseminated by laparoscopy using semen from East Friesland (white) rams. Embryos were subsequently flushed from the reproductive tract using standard surgical techniques. Embryo culture technique is very similar to that used in attempts at ES isolation, with 2 significant differences: (1) slightly later stage embryos, (blastodermic vesicles) are entered into culture and (2) undifferentiated ES-like early colonies are allowed to differentiate to generate rapidly dividing, permanent epithelial cell lines.

In contrast to Strelchenko *et al.*, *loc. cit.*, and Sims & First, *loc. cit.*, who used earlier stages, forty embryonic discs (EDs) at days 9 and 10 were dissected free of trophectoderm and cultured in groups of three to ten on inactivated primary mouse fibroblasts in 24 well culture plates. Culture medium was Glasgow's modified eagles medium (GMEM; Gibco, UK) supplemented with 10% foetal bovine serum (Gibco, UK) and 1000U/ml of the anti-differentiation agent, leukaemia inhibition factor (LIF). EDs were disaggregated by mild trypsinisation after 3-7 days (ie after attachment and evidence of outgrowth) and passaged onto a fresh feeder layer (passage 1). Within 7-10 days a proportion of wells showed small foci of undifferentiated cells. These were picked and passaged onto fresh feeders again in 24 well plates (passage 2 or P2). P2 cells expanded rapidly, losing their small, round, undifferentiated phenotype (Figure 1). At confluence, P2s were passaged into a single $25cm^2$ flask. four such lines were isolated and designated TNT/1 through 4. Three lines were frozen at passage 4.

The cell line TNT/4 was thawed and then cultured for a further 6 passages. Cells maintained a stable phenotype and failed to generate embryoid bodies when cultured in suspension. This differs markedly from ES cells and from the bovine cells of Strelchenko *et al.*, *loc. cit.*, which are characterised by their ability to form embryoid bodies and their undifferentiated morphology. There was no apparent change in phenotype when cultured in the absence of feeders. Experiments are continuing to characterise these cells for lineage specific markers.

**EXAMPLE 2: Generation of Lambs from TNT Nuclei**

Recipient oocytes were obtained following superovulation as per donors above, with the exception that no artificial insemination occurred. Unfertilised (Black Welsh Mountain and Scottish Blackface) MII oocytes were recovered in phosphate buffered saline (PBS) by flushing from the oviduct 31-33 h after gnRH injection. Recovered oocytes were washed in OCM medium (Gibco, UK) and transferred to medium TCM 199 (Gibco, UK). To remove the chromosomes (enucleation), oocytes were placed in TCM 199 containing 10% FBS, 7.5μg/ml cytochalasin B (Sigma, UK) and 5.0μg/ml Hoechst 33342 (Sigma, UK) at 37°C for 20 minutes. A small amount of cytoplasm from directly beneath the first polar body was removed with a finely drawn pipette and enucleation was confirmed by exposing the aspirated cytoplast to UV light and checking for the presence of a metaphase plate.

Oocytes were activated in a chamber consisting of two parallel platinum electrodes arranged 200 μm apart in a glass petri dish 9 cm in diameter. Oocytes were placed between the electrodes in 80 μl of activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.001 mM $CaCl_2$) in distilled

water. Activation was induced by a single DC pulse of 1.25 kV/cm for 80 μs.

Embryos were reconstructed by cell fusion (nuclear transfer from TNT cells) between 6 and 12 hours post activation (Campbell *et al.*, *Biol. Reprod.* **50** 1385-1393 (1994)). Fusion was carried out in the same chamber described above for activation, by application of a single AC pulse of 3V for 5 s followed by 3 DC pulses of 1.25 kV/cm for 80μs in activation medium. Reconstructed embryos were cultured in TCM 199 plus 10% FBS and 7.5 μg cytochalasin B for 1 hour at 37°C in 5% $CO_2$, were double embedded in 1% and then 1.2% agar (Difco) and then transferred to the ligated oviduct of unsynchronised Black Welsh Mountain ewes (temporary recipients for *in vivo* culture). After 6 days, recipient ewes were killed and the embryos were retrieved by flushing with PBS. Embryos were dissected from the agar. Those which had developed to morula or blastocyst stages were transferred to final synchronised recipients.

Seven reconstituted embryos were transferred into five final recipients, three of which returned to oestrus. The remaining two ewes yielded two liveborn lambs which displayed the white markings characteristic of the TNT lines (Table 1). One of these animals died shortly after birth and the remaining animals remain healthy at three months of age (Figure 2).

**ANNEX**

CLAIMS

1. An animal cell line derived from an embryonic disc of an ungulate blastodermic vesicle, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, cells of which cell line are totipotent for nuclear transfer.

2. An isolated animal cell which is derived from an embryonic disc of a ungulate blastodermic vesicle, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, and which is totipotent for nuclear transfer.

3. A cell line or cell as claimed in claim 1 or 2, wherein the animal is a placental mammal.

4. A cell line or cell as claimed in claim 1 or 2, wherein the animal is an ungulate.

5. A cell line or cell as claimed in claim 1 or 2, wherein the animal is a cow or bull, sheep, goat, water buffalo, camel or pig.

6. A cell line or cell as claimed in claim 1 or 2, which contains one or more transgenes.

7. A process for the preparation of an animal, the process comprising reconstituting an animal embryo by nuclear transfer from a TNT cell from a cell line as claimed in claim 1, or from a TNT cell as claimed in claim 2, allowing the embryo to develop to term and optionally breeding from the animal so formed.

8. A process as claimed in claim 7, wherein the TNT cell is genetically modified prior to embryo reconstitution.

9. A process as claimed in claim 8, wherein the TNT cell is genetically modified by electroporation, by viral transfection or by lipofection.

10. A process for the isolation of cells which are totipotent for nuclear transfer (TNT cells), the process comprising explanting at least part of the embryonic disc of an animal embryo at the blastodermic vesicle stage in the case of an ungulate, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, allowing the development of an ES-like colony of undifferentiated cells until they have acquired an enlarged, more epithelial phenotype and entering a cell or cells from such a colony into reproductive culture until required.

ANNEX

ABSTRACT

BIOLOGICAL MANIPULATION

Animal cells which are totipotent for nuclear transfer (TNT cells) can be isolated from the embryonic disc of the blastodermic vesicle of an ungulate, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species. TNT cells can be cultured *in vitro*, which allows cloning as desired and genetic manipulation, for example to introduce a transgene. Their nuclei can be transferred to suitable recipient cells and embryos reconstituted. In this way, animals of high genetic merit may be cloned and transgenic animals may be generated by mass transformation techniques across a broader range of species than is accessible with embryonic stem cell technology and without reliance on pronuclear microinjection.

FIGURE 1

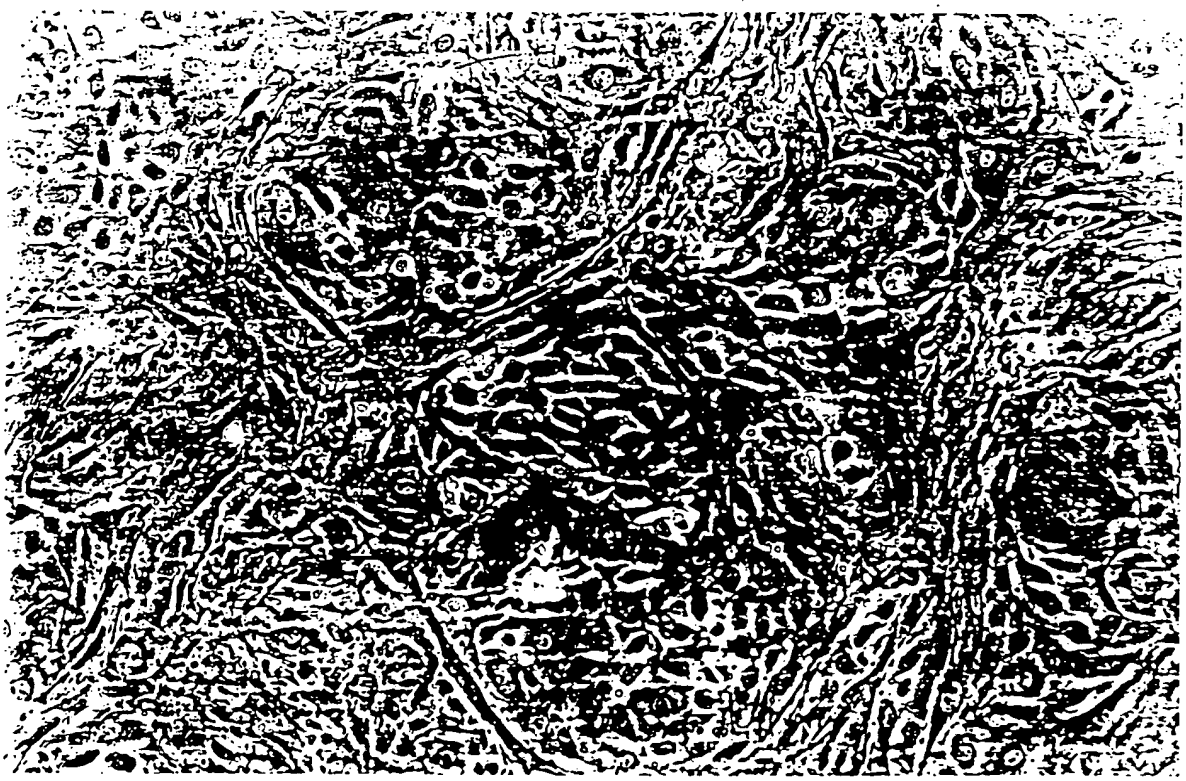

FIGURE 2